Effects of Dietary n-3 and n-6 Polyunsaturated Fatty Acids on Macrophage Phospholipid Classes and Subclasses

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This study examined the effects of n-3 and n-6 polyunsaturated fatty acid alimentation on murine peritoneal macrophage phospholipids. Mice were fed complete diets supplemented with either corn oil predominantly containing 18:2n-6, borage oil containing 18:2n-6 and 18:3n-6, fish/corn oil mixture containing 18:2n-6, 20:5n-3 and 22:6n-3, or fish/borage oil mixture containing 18:2n-6, 18:3n-6, 20:5n-3 and 22:6n-3. After two weeks, the fatty acid levels of glycerophosphoserines (GPS), glycerophosphoinositols (GPI), sphingomyelin (SPH), and f the glycerophosphocholine (GPC) and glycerophosphoethanolamine (GPE) phospholipid subclasses were determined. We found that mouse peritoneal macrophage GPC contain primarily 1-*O*-alkyl-2-acyl (range f r the dietary groups, 24.6–30.5 mol %) and 1,2-diacyl (63.2–67.2 mol %), and that GPE contains 1-*O*-alk-1′-enyl-2-acyl (40.9–47.4 mol %) and 1,2-diacyl (44.2–51.2 mol %) subclasses. In general, fish oil feeding increased macrophage 20:5n-3, 22:5n-3 and 22:6n-3 levels while simultaneously reducing 20:4n-6 in GPS, GPI, GPE and GPC subclasses except for 1-*O*-alk-1′-enyl-2-acyl GPC. Administration of 18:3n-6 rich diets (borage and fish/borage mixture) resulted in the accumulation of 20:3n-6 (2-carbon elongation product of 18:3n-6) in most phospholipids. In general, the novel combination of dietary 18:3n-6 and n-3 PUFA produced the highest 20:3n-6/20:4n-6 phospholipid fatty acid ratios. This study demonstrates that marked differences in the responses f macrophage phospholipid classes and subclasses exist following dietary manipulation. The reduction of 20:4n-6, while simultaneously increasing 20:3n-6 and n-3 PUFA levels, may be important in relation to the putative beneficial effects of 20:3n-6 and fish oil on macrophage eicosanoid and platelet activating factor (PAF) biosynthesis.
Lipids 25, 827–834 (1990).

The macrophage plays a central role in the immune system and is capable of executing or modifying a number of important biological functions (1). In addition to its ability to sequester, ingest and destroy microorganisms, this cell type can take part in both the amplification and suppression of immune responses (1,2). Macrophages are also known to secrete large quantities of arachidonic acid (20:4n-6) derived cyclooxygenase and lipoxygenase oxidative products (1,3). This is significant because the 20:4n-6 derived eicosanoids are capable of influencing many of the regulatory activities of macrophages (4,5). In addition, excess or imbalanced production of 20:4n-6 derived eicosanoids may exacerbate pathophysiological conditions such as asthma, arthritis and psoriasis (6,7). Therefore, an understanding of the mechanisms regulating macrophage eicosanoid production is vital in controlling inflammation and ameliorating pathophysiological states.

The cascade of 20:4n-6 release and metabolism is regulated in part by its specific phospholipid class distribution (8,9). Previous studies have proposed different classes of phospholipids, *i.e.*, glycerophosphocholines (GPC), glycerophosphoethanolamines (GPE), glycerophosphoinositols (GPI) and glycerophosphoserines (GPS) as sources of eicosanoid fatty acid precursors (10,11). It has been shown recently that considerable amounts of the phospholipid subclasses of GPC and GPE are present in alveolar macrophages (12). These subclasses of phospholipid are made up of 1-*O*-alkyl-2-acyl, 1-*O*-alk-1′-enyl-2-acyl and 1,2-diacyl species, each differing in the covalent linkage of the aliphatic chain at the *sn*-1 position of the glycerol backbone. The 1-*O*-alkyl and 1-*O*-alk-1′-enyl phospholipid subclasses are unique because they contain significantly higher levels of 20:4n-6 than diacyl species in certain cell types (12–14) and could be important pools of polyunsaturated fatty acids (PUFA) in macrophages. This is noteworthy since eicosanoid production can be regulated merely by altering the distribution of 20:4n-6 within the cell so that it is not released from phospholipids for eicosanoid synthesis (14). Thus, although a phospholipid may have a certain prototypic structure, it is in reality a family of related molecules with distinct metabolic and physical properties (15).

It is well established that alteration in the dietary content of fatty acids can modulate membrane bound receptors, enzyme activities, and eicosanoid production (16–18). One of the most interesting approaches to the dietary modification of the eicosanoid system has been the possible prophylactic role of dietary γ-linolenic acid (18:3n-6) and fish oil on thrombo-embolic (19,20) and chronic inflammatory disorders (21,22). Since borage oil, derived from the borage plant (*Borago officinalis*), contains large amounts of 18:3n-6 and fish oil contains large amounts of eicosapentaenoic acid (20:5n-3) and docosahexaenoic acid (22:6n-3), interest has focused on the possibility of competition between n-6 and n-3 fatty acids at the sites of membrane phospholipid storage and eicosanoid production (7,23). However, to date, detailed studies documenting the ability of dietary 18:3n-6 and fish oil to modify macrophage phospholipid classes and GPE and GPC subclasses are lacking.

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Abbreviations: 8-Anilino-1-naphthalenesulfonic acid ammonium salt; 20:4n-6, arachidonic acid; 20:3n-6, dihomogammalinolenic acid; 22:6n-3, docosahexaenoic acid; 22:5n-3, docosapentaenoic acid; 20:5n-3, eiocosapentaenoic acid; FAME, fatty acid methyl ester; 18:3n-6, γlinolenic acid; GPC, glycerophosphocholine; GPE, glycerophosphoethanolamine; GPI, glycerophosphoinositol; GPS, glycerophosphoserine; HPLC, high performance liquid chromatography; 18:2n-6, linoleic acid; PAF, platelet activating factor; PUFA, polyunsaturated fatty acids; SPH, sphingomyelin; TLC, thin-layer chromatography.

We have recently demonstrated (24) that fish oil feeding can suppress the activation of macrophage tumoricidal capability *in vitro*. These results suggest that fish oil feeding may play a regulatory role in host protection against neoplastic and infectious diseases. Prompted by these observations, we are now investigating how fish oil supplementation may act to influence host responsiveness. In order to address this complex issue, it is first necessary to determine the ability of n-3 fatty acids to alter macrophage membrane phospholipid profiles. Therefore, we report i) the levels of mouse peritoneal macrophage phospholipid subclasses; and ii) the effect of n-6/n-3 dietary fatty acid mixing on macrophage phospholipid class and subclass composition.

MATERIALS AND METHODS

Materials. Fatty acid methyl ester standards and monopentadecanoin were from NuChek Prep (Elysian, MN). Silica gel 60 plates and silica gel G were from E. Merck (Darmstadt, Federal Republic of Germany). Phospholipase C (*Bacillus cereus*, Type V) was from Sigma Chemical Co. (St. Louis, MO). 8-Anilino-1-naphthalenesulfonic acid ammonium salt was from Eastman Kodak (Rochester, NY). Benzoic anhydride and 4-dimethylaminopyridine were from Aldrich Chemical Co. (Milwaukee, WI). Corn and borage oil were donated by Traco Labs (Champaign, IL) and menhaden fish oil was donated by Zapata-Haynie (Reedville, VA). All chemicals were of high performance lipid chromatography (HPLC) grade.

Dietary treatments and macrophage isolation. Specific pathogen-free male C57BL/6 mice (Harlan, Indianapolis, IN) weighing 15–18 g were used. Mice were fed, *ad libitum*, one of four purified diets (Table 1) which were adequate in all nutrients (25). The diets were changed daily and varied only in the type of oil fed, *i.e.*, either corn, borage, fish/corn or fish/borage mixtures in a 1:1 ratio at 10% of the diet by weight, and were stored at −20°C. Under these conditions, fatty acid oxidative breakdown products are not detected (24,26). The fatty acid composition of the different diets is shown in Table 2. After two weeks of feeding the experimental diets, primary cultures of peritoneal macrophages were established from responsive cells as previously described (27). Selected cell monolayers were solubilized in 0.1 M sodium hydroxide for protein determination using the method of Lowry *et al.* (28).

Separation of phospholipid classes and subclasses. Macrophages were extracted by the method of Folch *et al.* (29). The individual phospholipid classes were separated by thin-layer chromatography (TLC) on silica gel 60 plates using chloroform/methanol/acetic acid/water (50:37.5:3.5:2, by vol.) as previously described (25). Bands were detected under ultraviolet light after spraying with 0.1% 8-anilino-1-naphthalenesulfonic acid ammonium salt (ANS). A known amount of monopentadecanoin as an internal standard was added to isolated GPS, GPI and SPH phospholipid classes prior to transesterification (25,30). The resultant fatty acid methyl esters (FAME) were further purified on silica gel G plates run in a toluene solvent system, detected using ANS, and extracted using methanol/hexane/water (1:1:0.5, by vol.) prior to gas chromatographic analysis. The isolated choline (GPC) and ethanolamine (GPE) glycerophospholipid bands were extracted using chloroform/methanol/water (5:5:1, by vol.) followed by the addition of 2.25 mL chloroform and 1 mL 50 mM Tris buffer, pH 9.0 (31). The upper aqueous layer was discarded and the solvent from the lower chloroform phase was removed under N_2. The isolated GPC and GPE fractions were converted to benzoate derivatives following phospholipase C hydrolysis (32,33). Briefly, approximately 0.4 mg of phospholipid was suspended in 2 mL peroxide-free diethyl ether containing 0.005%

TABLE 1

Composition of Experimental Diets

Ingredient[a]	Amount (g/100 g diet)
Oil[b]	10.00
Casein (vitamin free)	20.00
DL-methionine	0.30
Sucrose	44.00
Corn starch	14.98
Cellulose	6.00
Mineral mix[c]	3.50
Vitamin mix[d]	1.00
Choline chloride	0.20
t-Butylhydroquinone[e]	0.02

[a]All dietary components were purchased from U.S. Biochemicals (Cleveland, OH), except where noted.
[b]All diets provide approximately 22% energy from lipid.
[c]Provided at the following amount in grams/kilogram of salt mix as per AIN 76 mixture: $CaHPO_4$, 500.0; NaCl, 74.0; K-citrate, 220.0; K_2SO_4, 52.0; MgO, 24.0; manganous CO_3, 3.5; ferric citrate, 6.0; $ZnCO_3$, 1.6; $CuCO_3$, 0.3; KIO_3, 0.01; Na_2SeO_3, 0.01; and $CrK(SO_4)_2$, 0.55.
[d]Provided at the following amount in grams/kilogram of vitamin mix (except as noted) as per AIN 76 mixture; thiamin HCl, 0.6; riboflavin, 0.6; pyridoxine HCl, 0.7; nicotinic acid, 3.0; Ca pantothenate, 1.6; folic acid, 0.2; biotin, 0.02; cyanocobalamin, 1 mg/kg; retinyl palmitate, 1.6 (250,000 I.U./g); dl-α-tocopheryl acetate, 20 (250 I.U./g); cholecalciferol, 2.5 mg (400,000 I.U./g); and menaquinone, 5.0 mg.
[e]Eastman Kodak Chemicals (Rochester, NY).

TABLE 2

Fatty Acid Composition of Diets

Fatty Acid[a]	Corn	Borage	Fish[b]/Corn	Fish[b]/Borage
14:0	tr	0.3	4.5	4.5
16:0	12.1	10.3	14.1	13.4
16:1n-7	0.2	0.1	6.1	5.9
18:0	1.7	3.2	2.6	3.5
18:1n-9	25.7	14.7	16.4	10.8
18:2n-6	59.0	39.2	30.6	20.4
18:3n-6	tr	25.6	0.3	13.0
20:4n-6	tr	tr	0.4	0.4
20:5n-3	tr	tr	7.6	6.8
22:5n-3	tr	tr	1.0	1.0
22-6n-3	tr	tr	3.9	4.1

[a]Values are expressed as mg/100 mg total fatty acids present. Only the major fatty acids are presented (tr=trace amounts, less than 0.1%).
[b]Menhaden fish oil.